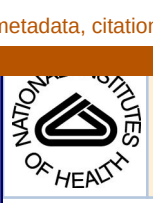

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Evidence for an unusual transmembrane configuration of AGG3, a Class C Gγ Subunit, of Arabidopsis

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SUMMARY

Heterotrimeric G proteins are crucial for the perception of external signals and subsequent signal transduction in animal and plant cells. In both model systems, the complex is comprised of one Gα, one Gβ and one Gγ subunit. However, in addition to the canonical Gγ subunits (Class A), plants also possess two unusual, plant-specific classes of Gγ subunits (Classes B and C) not yet found in animals. These include Gγ subunits lacking the C-terminal CaaX motif (Class B) which is important for membrane anchoring of the protein, and thus give rise to a flexible subpopulation of Gβ/γ heterodimers that is not necessarily restricted to the plasma membrane. Even more interesting, plants also contain Class C Gγ subunits which are twice the size of canonical Gγs, with a predicted transmembrane domain, and a large cysteine-rich, extracellular C-terminus. However, neither the presence of the transmembrane domain nor the membrane topology has been unequivocally demonstrated. Here, we provide compelling evidence that AGG3, a Class C Ggamma subunit of Arabidopsis, contains a functional transmembrane domain, which is sufficient but not essential for plasma membrane localization, and that the cysteine-rich C-terminus is extracellular.

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Accession numbers Sequence data are available at GenBank under the following accession numbers: At5g20635.1 (AGG3), At4g16480.1 (INT4), At3g26090.1 (RGS1), At2g26300 (GPA1), and At4g34460 (AGB1).

Keywords

Arabidopsis Gγ subunit 3, AGG3; membrane topology; Heterotrimeric G protein; *Arabidopsis thaliana*

INTRODUCTION

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are major components of the transmembrane signaling system in eukaryotes and mediate various physiological responses (Urano *et al.* 2013). G proteins are comprised of one alpha (Gα), one beta (Gβ) and one gamma (Gγ) subunit. Gα binds and hydrolyses guanosine triphosphate (GTP) thereby determining the active-inactive state of the heterotrimeric G protein complex, while the Gβ subunit possesses a 7-bladed propeller structure and forms a functional heterodimer with the Gγ subunit. Upon activation of the G protein, the GTP-bound Gα subunit and the Gβ/γ dimer dissociate from each other to subsequently modulate distinct downstream effectors (Cabrera-Vera *et al.* 2003, Offermanns 2003).

In contrast to the canonical mechanisms described in animals and fungi (Wess 1997), activation of plant G protein signaling in Arabidopsis follows a different course of action and involves the internalization of the negative regulator AtRGS1, which functions as a 7-transmembrane, receptor-like GTPase-activating protein (GAP) and keeps Gα in its inactive, GDP-bound state (Chen and Jones 2004, Chen *et al.* 2003, Johnston *et al.* 2007). Furthermore, the steady-state level of G protein subunits in plants is low and probably rate limiting to some aspects of G signaling (Fu *et al.* 2014). Because cereals lack 7-transmembrane RGS proteins, another mechanism for regulation of the active state of G signaling must exist. While the human genome for instance encodes 16 Gα, five Gβ and 12 Gγ subunit (Simon *et al.* 1991), only one Gα (GPA1), one Gβ (AGB1), and three Gγ (AGG1-3) isoforms are present in *Arabidopsis thaliana* (Chakravorty *et al.* 2011, Ma *et al.* 1990, Mason and Botella 2000, Mason and Botella 2001, Weiss *et al.* 1994). Thus, functional selectivity of the heterotrimer in plants is determined by the Gγ subunits in Arabidopsis, rice, and probably all plants (Thung *et al.* 2013, Trusov *et al.* 2007, Trusov *et al.* 2008).

The structure of the animal Gγ subunit is well understood (Gautam *et al.* 1998, Robishaw and Berlot 2004). By means of the N-terminal γ domain, the Gγ subunit forms a coiled-coil structure with its Gβ partner (McCudden *et al.* 2005, Pellegrino *et al.* 1997), and the C-terminus contains a CaaX motif (C = Cys; a = aliphatic amino acid; X = any amino acid) that is prenylated thus keeping the protein tethered to the P face of the plasma membrane (PM) (Chakravorty and Botella 2007, Simonds *et al.* 1991, Zeng *et al.* 2007). All 12 human Gγ subunits represent small membrane-associated proteins; however no animal Gγ subunit to date is known to have a transmembrane or an extracellular domain. In contrast, plants have at least three structurally-distinct classes of Gγ subunits; those currently known are designated class A, B, and C (Fig. 1a) (Trusov *et al.* 2012). *Arabidopsis* AGG1 and AGG2 belong to class A and are structurally similar to the canonical Gγ subunits found in animal cells. Class B Gγ subunits possess the N-terminal γ domain, but lack the CaaX motif.

Therefore the resulting subpopulation of Gβ/γ dimers may not be delimited to the PM. Representatives of this class are not found in *Arabidopsis*, but are present in most other flowering plants (Trusov *et al.* 2012), as exemplified by RGG2 from rice (Kato *et al.* 2004). AGG3 belongs to class C Gγ subunits that possess special features compared to all other Gγ subunits. With 251 amino acids, AGG3 is twice as large as AGG1 and AGG2 (Chakravorty *et al*. 2011). AGG3 contains a typical N-terminal γ domain, but may also possess a transmembrane domain (TMD) and the cysteine-rich C-terminus may be extracellular (Botella 2012, Li *et al.* 2012). If confirmed, this unusual Gγ membrane topology is significant since it not only defines a new prototype of Gγ subunits but also implies that class C Gγ subunits have an extracellular function. Extracellular functionality for a Gγ subunit is unprecedented. Importance of the cysteine-rich C-terminus for AGG3 function in plants was suggested in our previous work, where we demonstrated that the phenotype of *agg3-3* knock-out mutants is not rescued by complementation with a C-terminal-truncated AGG3 protein (Chakravorty *et al.* 2011). However, these previous studies do not conclusively address the question of whether AGG3 possesses a TMD. Localization studies in stable *Arabidopsis* lines over-expressing translational GFP fusions of AGG3 suggested a PM localization of the protein, although fusion proteins were also detected in various other subcellular compartments including the Golgi and the nucleus (Chakravorty *et al*. 2011, Li *et al*. 2012). While a function of the putative TMD in the subcellular localization of AGG3 was postulated, the previous data did not fully support this because deletion of the transmembrane region did not abolish the PM localization of the protein entirely (Chakravorty *et al*. 2011, Li *et al*. 2012). Considering the uniqueness and the physiological importance of a Gγ subunit with a transmembrane domain, it is critical to further assess the proposed Gγ membrane topology of the class C Gγ subunit, AGG3.

Using different independent approaches, we provide strong evidence that AGG3 represents a membrane protein with an extracellular cysteine-rich C-terminus. A possible role for class C Gγ subunits in the perception of external signals and environmental cues is discussed.

RESULTS AND DISCUSSION

Three classes of Gγ subunits

The *agg1/agg2/agg3* triple mutant shares the known *agb1-2* mutant phenotypes (Thung *et al.* 2012) and extensive homology searches failed to identify class B, or additional class A and class C Gγ subunits in the *Arabidopsis* genome (Trusov *et al*. 2012) suggesting that there are only three Gγ subunits present in *Arabidopsis*. However, due to the limited homology and possible unexplored phenotypes of null mutations in the Gβ subunit, we sought biochemical evidence that *Arabidopsis* has only the three known Gγ subunits, and that no further subunits dimerize with Gβ. From studies on animal G proteins, it is well known that the formation of a functional Gβ/γ dimer is crucial for the stability and localization of mammalian Gβ subunits (Dingus *et al.* 2005, Mervine *et al.* 2006). Based on this knowledge, we studied the stability of AGB1 proteins in the Gγ triple knockout mutant background (*agg1/agg2/agg3*). Proteins extracted from leaves of WT plants and different G protein mutants (*rgs1-2*, *gpa1-4*, *agb1-2*, *agg1/agg2* or *agg1/agg2/agg3*) were fractionated into membrane and soluble protein fractions, subjected to SDS-PAGE and detected using

anti-AGB1 antibodies (Fig. 1b). Because of the low abundance of the endogenous G protein subunits, detection by immunoblot required sample overloading. AGB1 was detected in the membrane fraction from WT plants. The traceable amount of Gβ protein was unchanged in *rgs1-2* and *gpa1-4* mutants, reduced in *agg1/agg2* double mutants, and severely decreased in *agg1/agg2/agg3* triple mutants. This result demonstrates that at least one of the three known Gγ subunits is needed to form a functional Gβγ dimer, and suggests that no further Gγ subunits are present in *Arabidopsis*, which is consistent with the previous conclusion (Thung *et al.* 2012; Trusov *et al*. 2012). In addition, these results indicate that loss of all three Gα subunits destabilizes the Gβ subunit as indicated by the reduced AGB1 signal in the soluble fraction. The Gα protein (AtGPA1), whose localization is independent of Gβ or Gγ, was used as a control. As expected AtGPA1, was absent in *gpa1-4* mutants, but could be detected in comparable amounts in the membrane fractions from *rgs1-2*, *agb1-2*, *agg1/agg2* and *agg1/agg2/agg3* plants (Fig. 1b).

Membrane topology of AGG3

Like other members of plant class C Gγ subunits (Fan *et al.* 2006, Mao *et al*. 2010), AGG3 contains a predicted TMD with a weak TM score (Fig. S1). A comprehensive set of topology prediction algorithms equivocally returned either weak or strong TM scores for plant class C proteins (Fig. S1). It was previously claimed that this postulated TMD is important for the subcellular localization of AGG3. However, inconsistent with this conclusion, deletion of the corresponding domain did not abolish PM localization (Li *et al.* 2012). As a TMD in a Gγ subunit is unprecedented, it is critical to further investigate this topology.

We first assessed the existence of a potential extracellular domain in AGG3 using the split-ubiquitin membrane-based yeast two-hybrid system (Stagljar *et al.* 1998). The N-terminal half of the ubiquitin I13G mutant (Nub_G) was fused either to the N- or the C-terminus of AGG1, AGG2 or AGG3, respectively, while the C-terminal half (Cub) was fused to the C-terminus of AGB1, and vice versa. If the C-terminus of AGG3 is extracellular, a C-terminal fusion will place the ubiquitin fragment outside the cell rendering it unable to complement growth. An N-terminal AGG3-fusion to the N-terminal half of WT ubiquitin (Nub_{wt}), which spontaneously interacts with Cub in yeast cells (Stagljar *et al*. 1998) served as the positive control. Yeast strains co-expressing Nub_G-AOC3, AOC3-Nub_G, or free Nub_G (empty vector) were used as negative controls.

There was a clear difference in the growth of AGG1-Cub and AGG2-Cub fusions compared to AGG3-Cub fusions. The Cub fragment contains the PLV transcription factor which is cleaved and released upon Nub-Cub reformation (Stagljar *et al*. 1998). In some cases, soluble proteins that are capable of localizing to the nucleus result in autoactivation independent of Nub-Cub reformation and PLV cleavage. In our experience, AGG1-Cub and AGG2-Cub cause autoactivation of the split-ubiquitin system, as exemplified by growth with the Nub_G negative control, even on the SD + 500 μM methionine high stringency medium (Fig. S2, subpanels e and k). Fusion of the Cub fragment to the C-terminus of AGG1 or AGG2 may cause disruption of the C-terminal isoprenylation motif, and therefore, loss of membrane association, which could contribute to the observed autoactivation. In

contrast, AGG3-Cub combined with positive controls (Nub_{WT} combinations), or known interactors (Nub_G-AGB1 or AGB1-Nub_G) did not result in growth, even on low stringency SD + 0 μM methionine medium (Fig. S2, subpanels m, n, o, p and r). Therefore there is an inherent difference between AGG1-Cub/AGG2-Cub, and AGG3-Cub in the split-ubiquitin system, which renders AGG3-Cub non-functional, and is consistent with but not conclusive evidence for an extracellular C-terminal topology.

When Nub-Gγ fusions were tested, Nub_G-AGG1, Nub_G-AGG2, AGG1-Nub_G and AGG2-Nub_G fusions all resulted in yeast growth when combined with AGB1-Cub, as expected (Fig. 2a, subpanels a-h). Interactions between Nub_{wt}-AGG3/AGB1-Cub or Nub_G-AGG3/AGB1-Cub also complemented growth (Fig. 2a, subpanels i and j), as would be expected for an intracellular N-terminus of AGG3. In contrast, no growth was detected when the ubiquitin fragments were attached to the C-terminus of AGG3 (AGG3-Nub_G/AGB1-Cub or AGG3-Nub_{WT}/AGB1-Cub). This result is consistent with the hypothesis that AGG3 contains a transmembrane span with the C-terminus being extracellular and thus unavailable for interaction in the split-ubiquitin assay (Fig. 2a, subpanels k and l).

To rule out the possibility that negative results derive simply from a lack of expression of AGG3-Nub fusions, we performed a western blot using an anti-HA antibody, targeting the HA epitope tag on the C-terminus of all Nub fusions. We observed that X-Nub_{WT} fusions were expressed considerably more weakly than X-Nub_G fusions (Fig. 2b). The weak expression of AOC3-Nub_{WT} and lack of expression of AGG3-Nub_{WT} (Fig. 2b) likely explain why these 'positive control' fusions did not result in growth when combined with AGB1-Cub (Fig. 2a, panels l and p). However, when AGB1-Cub was combined with a strong interactor (e.g. AGG1-Nub_{WT} or AGG2-Nub_{WT}), yeast growth (Fig. 2a, panels d and h) demonstrates that weak expression is sufficient to result in complementation in the case of a positive interaction. Furthermore, the expression of AGG1-Nub_G/AGG2-Nub_G was comparable to the expression level of AGG3-Nub_G, yet AGG1-Nub_G/AGG2-Nub_G resulted in growth while AGG3-Nub_G did not. Therefore, despite being a strong AGB1-interactor, and expressed at levels similar to AGG1-Nub_G and AGG2-Nub_G, AGG3-Nub_G displays some inherently different characteristics, consistent with an intracellular N-terminus, and extracellular C-terminus. Additionally, we performed a western blot with anti-AGB1 antibody and confirmed that the AGB1-Cub fusion was expressed in the relevant X-Nub_G and XNub_{WT} samples included in Fig. 2b (Fig. S3).

As an independent method to determine the membrane topology of AGG3, we measured the relative fluorescence intensities of stably transformed *Arabidopsis* plants expressing GFP fused to either the N- or the C-terminus of AGG3. Our approach was based on the observation by Zheng et al. that fluorescence of apoplastic GFP is subject to quenching by low pH (Zheng *et al.* 2004). Using stable expression we show that when GFP was fused to the C-terminus of AGG3, but not when it was fused to the N-terminus of AGG3, the GFP signal was subject to quenching by low pH (Fig. 3), indicating the topology of AGG3 as a membrane protein with an extracellular C-terminus.

The mechanism for membrane anchoring of AGG3

Conventional membrane-bound Gγ subunits (class A; Fig. 1a) contain C-terminal prenylation motifs (CaaX) which are essential for PM anchoring of the proteins. AGG3 contains a C-terminal CaaX motif (Chakravorty *et al*. 2011), although approximately half of the class C proteins available in the databases lack a CaaX motif (Trusov *et al*. 2012). Furthermore, the presence of a putative TMD leaves the function of the CaaX motif unclear. It was reported that deletion of the AGG3 TMD was not sufficient to completely abolish the PM localization of AGG3 (Li *et al*. 2012). Thus, it might be possible that prenylation of a cryptic CaaX motif substitutes for the loss of the TMD to keep the AGG3ΔTMD mutant at the PM.

The classical experiment for assessment of protein topology involves the expression of protein truncations and domain swaps in a heterologous system and topology probing using extracellular proteolysis (Lorenz *et al.* 2006, Wunder *et al.* 2010). We attempted to express plant Gγ subunits in mammalian HEK293 and COS7 cells, but even after extensive codon-optimization we failed to reproducibly obtain sufficient protein levels for a robust conclusion. In our hands, only expression in plant protoplasts was successful.

Using transient expression in *Arabidopsis* mesophyll protoplasts, we tested the ability of distinct protein domains to localize AGG3 to the PM. A set of different AGG3 mutants was created (Fig. 4a; Fig. S4) and the subcellular localization of the resulting proteins analysed using N-terminal GFP fusions. Additionally, co-localization studies with a PM-localized *myo*-inositol transporter (INT4) (Schneider *et al.* 2006) were performed, to differentiate between soluble and membrane-bound fusion proteins (Fig. 4b). Protoplasts transiently over-expressing *35S::GFP* were used as control for soluble GFP (Fig. 4c; left). As shown in Fig. 4c, when soluble GFP was expressed, a ring of fluorescence with thicker and thinner sections was detected around the cell (Fig. 4c; blue arrows) and GFP fluorescence was also detectable in the gaps between chloroplasts indicating cytosolic localization. Upon co-localization with INT4-RFP, no overlap of the green (yellow arrow) and red (white arrow) fluorescence was observed (Fig. 4c; right). As a positive control for PM-localized GFP fusion proteins, protoplasts over-expressing *35S::RGS1-GFP* showed an even ring of fluorescence around the cell (Fig. 4d; left). Protoplasts co-expressing *35S::RGS1-GFP* and *35S::INT4-RFP* showed complete overlap of the green and red fluorescence (Fig. 4d; right; orange arrow).

Unlike results from previous reports (Chakravorty *et al*. 2011, Li *et al*. 2012), GFP-AGG3 was exclusively localized at the PM of the protoplasts and no fluorescence was detected in the Golgi apparatus or the nucleus (Fig. 4e). As expected for plasma membrane proteins (Bassham *et al.* 2008), AGG3-GFP fusion proteins containing the TMD were often detected in these intermediary compartments in subcellular localization studies.

Amino acid substitutions leading to mutation of the C-terminal CaaX-motif (AGG3ΔCaaX) did not affect the subcellular localization of AGG3 and GFP fluorescence was still observed solely at the PM (Fig. 4f). Deletion of the AGG3 transmembrane region (AGG3ΔTMD) partially redistributed the GFP fusion protein with some GFP fluorescence clearly detectable in the cytosol (Fig. 4g; yellow arrow). However, the majority of GFP-AGG3ΔTMD was still

localized at the PM (Fig 4g, orange arrow). This result is consistent with the previous observation by Li *et al*. of the TMD not being essential for membrane localization of AGG3 (Li *et al*., 2012). One possible explanation is that the cryptic CaaX motifs (Fig. S4) which are normally not exposed to the farnesyl transferase complex in the cytosol, become farnesylated on an AGG3 molecule that lacks a transmembrane span, and then enable plasma membrane association via this lipid modification. Similarly, AGG3 proteins lacking both the TMD and the C-terminal CaaX motif, but still containing the large C-terminal cys-rich domain (AGG3ΔCaaXΔTMD) were partitioned to some extent to the PM (Fig. 4h; orange arrow).

Consistent with the idea of residues 108-125 forming a TMD, removal of the entire C-terminus but with retention of this putative TMD (AGG3ΔCT) did not influence the PM localization of AGG3 (Fig. 4i). Only deletion of both the C-terminus and the putative TMD (AGG3ΔTMDΔCT) caused a redistribution of the AGG3 mutant protein to the cytosol (Fig. 4j).

AtGPA1 and AGB1 are not needed for correct localization of AGG3

Our localization studies in *Arabidopsis* protoplasts revealed that AGG3 is localized at the PM even when the putative TMD and the C-terminal CaaX motif are removed. Therefore some other mechanism seems to be involved in plasma membrane association of this AGG3 mutant protein. However, removal of the cys-rich C-terminus in addition to the transmembrane region abolished the PM localization completely. As cys-rich regions are often involved in protein-protein interactions and complex formation (Labunskyy *et al.* 2005, Okada *et al.* 1999, Voorberg *et al.* 1991), it is possible that other subunits of the heterotrimeric G protein complex are involved in the subcellular localization of AGG3. In accordance with this hypothesis, co-infiltration of *Nicotiana benthamiana* leaves with *35S::AGB1* and *35S::AGG3-GFP* increased the total amount of measurable GFP fluorescence at the PM significantly (Chakravorty *et al*. 2011). To further investigate a putative role for AGB1 and GPA1 in the targeting of AGG3, we performed localization studies in protoplasts from *gpa1-4/agb1-2* double mutants.

Protoplasts expressing *35S::GFP* or *35S::RGS1-GFP* were used as controls for soluble or PM-localized fusion proteins, respectively (**Fig. 5a** and **5b**). As observed in WT protoplasts, AGG3 localized to the PM of *gpa1-4/agb1-2* mutant cells; this was confirmed via co-localization experiments with the PM marker INT4-RFP (Fig. 5c). Since DEP1, a rice homolog of AGG3, directly or indirectly interacts with the rice Gα subunit (Sun *et al.* 2014), it was necessary to determine if localization of AGG# required the AtGPA1 or AGB1. The localization of the various tested AGG3 mutants did not change in the Gα/β mutant background compared to their subcellular localization in WT protoplasts (**Fig. 5d** to **5h**). These results indicate that neither GPA1 nor AGB1 are involved in the membrane association of AGG3. Moreover, the localization of AGG3 or AGG3-CaaXΔTMD was also not affected in the *gpa1-4/agb1-2/agg1/agg2* quadruple mutant background (Fig. S5 and S6).

AGG3 is a type II membrane Gγ subunit

In summary, despite weak indication from topology-prediction algorithms, the results from our studies obtained by independent and complementary experimental approaches, are all consistent with the hypothesis that AGG3 contains a TMD with a large extracellular cys-rich C-terminus. Subcellular localization studies with various translational GFP fusions of different AGG3 mutants revealed that both the residues 108-125, encompassing the postulated TM region and the C-terminal extracellular half are involved in PM anchoring of AGG3. However, neither the extracellular domain of AGG3 nor the CaaX motif was sufficient to delimit the protein to the plasma membrane. Elaboration of this result and further conclusions are made in the next subsection.

To address the question of AGG3 membrane topology, split-ubiquitin-based yeast complementation assays and pH-sensitive fluorescence quenching assays were performed. Yeast growth facilitated by restoration of a functional ubiquitin molecule was only observed when the split half of ubiquitin was attached to the N-terminus but not the C-terminus of AGG3, and therefore (in agreement with the predicted membrane topology), was presented on the cytosolic side. In addition, when a GFP tag was placed at the C-terminus of AGG3 but not at its N-terminus, pH sensitivity of fluorescence was observed in the quenching assay indicating that the C-terminal domain is apoplastically located and further supporting the presence of a single TMD.

Moreover, in contrast to intracellular proteins, extracellular proteins (or protein domains) contain a high percentage of cysteines and half-cystines that form disulfide bridges (Fahey *et al.* 1977). The half-cystine content of the hypothesized extracellular domain (residues 128-251) of AGG3 is about 34%, which strongly suggests that this domain is extracellular. This observation in combination with the experimental evidence provided in this study, strongly support the hypothesis that AGG3 has the membrane topology of a typical type II membrane protein.

AGG3 is the prototype of class C Gγ subunits

A large extracellular domain in a Gγ subunit raises the interesting possibility that additional extracellular signaling is mediated, at least in part, through the Gβ/γ dimer. This possibility is intriguing and, if true, is unprecedented. Alternatively or in addition, the extracellular cys-rich region of AGG3 may play a structural or stabilizing role in the formation of protein complexes in the apoplast. Restricting Gβ/γ dimers, thus G protein signaling as a whole, to microdomains of the PM raises another possibility for a G protein signaling control mechanism. As G proteins influence the sugar profile of cell walls (Klopffleisch *et al.* 2011), we speculate that sugar composition of the cell wall regulates G signaling in a feedback loop. If AGG3 is important for cell wall composition, we speculate that the AGG3 extracellular domain may directly contact wall components as a mechanism to assess composition.

EXPERIMENTAL PROCEDURES

Plant material and reagents

Stably-transformed *Arabidopsis* lines expressing GFP-tagged AGG3 are described in Chakravorty *et al*., 2011.

Phylogenetic analyses

Full-length protein sequences of Gγ subunits from *A. thaliana* (At3g63420.1, At3g22942.1 and At5g20635.1), *V. vinifera* (GSVIVT01018076001, GSVIVT01015067001 and GSVIVT01015067001), *S. bicolor* (Sb01g014060.1, Sb04g003060.1, Sb01g032830.1, Sb02g025860.1 and Sb07g022330.1) and *H. sapiens* were collected from NCBI or JGI proteome database. The sequences were aligned by the CLUSTAL W algorithm implemented in MEGA5.0 and regions containing 70% or more gaps were deleted from the aligned sequences. The maximum-likelihood tree was created using the Jones-Taylor-Thornton model (Jones *et al.* 1992) with bootstrap analysis of 500 replicates.

Protein extraction and immunoblot analyses

Leaves from 7-week-old *Arabidopsis* WT plants or G protein null mutants were collected, frozen, and ground in liquid nitrogen. The ground leaves were suspended in extraction buffer (50 mM Tris-HCl, pH 8.0; 10% glycerol; 10 mM β-mercaptoethanol) containing protease inhibitor cocktail (Sigma Aldrich, USA) and centrifuged for 60 min at 27,000 rpm in a Beckman centrifuge (TLS-55 rotor). The supernatants were collected and kept as cytosolic protein fractions, while the pellets were solubilized in extraction buffer containing 1% NP-40 or 2% SDS and used as membrane fractions. Gα and Gβ proteins were detected by immunoblot analyses using anti-AtGPA1 or anti-AGB1 antibodies. The volumes of the samples were adjusted according to dilution during preparation in order that the level of G protein subunit detected by the antiserum in the soluble and membrane fractions are directly comparable. Antisera to GPA1 were prepared as described in the supplemental section of Chen et al (2003). Antisera against AGB1 was prepared in rabbits by Open BioSystems (Huntsville, AL USA) using the peptide TETVNNLRDQLRQRRLQLK as the antigen.

Split-ubiquitin membrane-based yeast assays

The mating-based split ubiquitin system (mbSUS) was used to examine the interaction between AGB1 and Gγ subunits (AGG1, AGG2, AGG3). The N-terminal half of the ubiquitin I13G mutant (Nub_G) was fused either to the N- or the C-terminus of AGG1, AGG2 or AGG3. Nub and Cub constructs were generated by transferring sequence verified clones from pCR®8/GW/TOPO® (Life Technologies; http://www.lifetechnologies.com) into the indicated yeast expression vector (Lalonde *et al.* 2010) by Gateway cloning methods. The Nub_{WT}-fusion proteins were used as positive controls, and empty vector containing Nub_G only was used as a negative control. When expressed from an empty vector, unlike NubWT, NubG does not spontaneously bind to the Cub fragment, and therefore the PLV transcription factor is not released in X-Cub/Nub-EV combinations (Obrdlika *et al.* 2004). Each entire set of interaction assays was repeated twice, and combinations including AGG3 were repeated five times. Mating and growth conditions were performed as described (Obrdlik *et al.* 2004).

Expression of Nub constructs was verified by immunoblot, using an anti-HA (clone 3F10) peroxidase conjugated antibody (Roche Applied Science; www.roche-applied-science.com).

Generation of expression plasmids

All AGG3 sequences were amplified with primers introducing flanking BspHI sites for subsequent cloning into protoplast expression vectors. The full-length coding sequence (CDS) for the WT AGG3 protein was amplified using the primers *AGG3-5-BspHI* (TCATGAGTGCTCCTTCTGGCGGTG) and AGG3-3-BspHI (TCATGACGAAAGCTAAACAACAAGG). To generate an AGG3 mutant with degenerated prenylation motif (ΔCaaX), the full length CDS was amplified using the primers *AGG3-5-BspHI* (TCATGAGTGCTCCTTCTGGCGGTG) and *AGG3-CaaX3-BspHI* (TCATGACGAAAGCTAAAGAAGAAGG), causing amino acid exchanges $C_{247}S$ and $C_{248}S$ in the resulting protein sequence. To generate the C-terminal truncation mutants AGG3ΔCT and AGG3ΔTMDΔCT the forward primer *AGG3-5-BspHI* was combined with the reverse primer *AGG3-dCT-BspHI-R* (TCATGACTGCTTGGCAGCAACAGCAGAAACTC) or *AGG3-dCT-dTM* (TCATGACTGCTCTTCGACTTTTTCGTTGTGCAG), respectively. AGG3 and AGG3-CaaX mutants lacking the putative TMD (residues 108-125) were amplified with the primers *AGG3-5-BspHI* and *AGG3-3-BspHI* or AGG3-CaaX3-BspHI, respectively, from plasmid DNA coding for $AGG3^{\Delta 108\text{-}125}$ (Li *et al.* 2012). The full-length CDS of *RGS1* was amplified using the primers *RGS1-5-NcoI* (CCATGGCGAGTGGATGTGCTCTACATGGTGGTTG) and *RGS1-3-NcoI* (CCATGGCACCGGGACTACTGCATCTGGAACTCTTTGAC). The resultant sequences were then cloned into the protoplast expression vectors pCS120 for C-terminal GFP fusions (Dotzauer *et al.* 2010) or pSS87 for N-terminal GFP fusions (Schneider *et al.* 2012). As a PM marker for co-localization studies the *myo*-inositol transporter AtINT4 (At4g16480) carrying a C-terminal RFP fusion was used (Wolfenstetter *et al.* 2012).

Mesophyll protoplast transformation

Protoplasts from *Arabidopsis* Col-0 WT plants and *gpa1-4/agb1-2* mutants were generated as described (Drechsel *et al.* 2011) and transformed as described (Abel and Theologis 1994). Forty-eight hours after transformation, the subcellular localization of GFP and RFP fusion proteins was analyzed using a confocal laser-scanning microscope (Zeiss LSM 710 Duo; Jena, Germany). Only protoplasts with low or intermediate fusion protein expression were analyzed. Excitation of the fluorophores was performed with laser light of 488 nm (GFP) or 560 nm (RFP) wavelength. Detection windows ranged from 493 nm to 531 nm for GFP and from 573 nm to 641 nm for RFP and mCherry. Chloroplast autofluorescence was detected from 689 nm to 758 nm. All images were processed with the Zen 2009 Confocal Software.

GFP fluorescence quantification

For the study of pH effects on GFP fluorescence, GFP fluorescence from root epidermal cells of one-week-old *Arabidopsis* seedlings (grown on 1/2 MS medium with pH adjusted to either 5.5 or 8.1 using MES or HEPES, respectively) was imaged and quantified as described (Sheahan *et al.* 2004) but without optical sectioning. GFP fluorescence was

imaged with a confocal laser-scanning microscope (LSM 510; Zeiss) equipped with a 40X C-Apochromat water-immersion objective (NA 1.2; Zeiss) using a 488 nm Argon laser and BP500-530IR filter. GFP fluorescence intensity was quantified from mid-plane cell sections of a minimum of 200 cells. Fluorescence values were normalized to account for an observed pH-dependent change in autofluorescence of Col-0 seedlings captured with the applied image acquisition settings. Normalization was therefore performed by subtracting the ratio of fluorescence intensities in Col-0 plants at pH 8.1 and pH 5.5 from the same ratio in GFP-AGG3 or AGG3-GFP plants and then multiplying this value by the fluorescence intensity at pH 5.5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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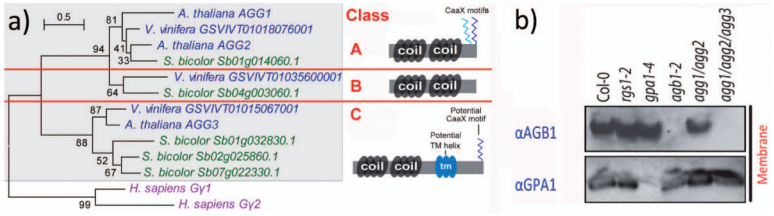

Figure 1. Three different Gγ subunits can be found in flowering plants

(a) Maximum-likelihood tree of representative Gγ subunits from *Arabidopsis thaliana* (dark blue), *Vitis vinifera* (light blue), *Sorhum bicolor* (green) and *Homo sapiens* (magenta). The bootstrap support values are shown near each branch. A schematic overview of the domain structures of the three different plant Gγ subunit classes is given on the right. **(b)** Immunoblot analyses of endogenous levels of Arabidopsis Gα and Gβ subunits in membrane and soluble fractions extracted from wild type (Col-0) or G protein mutant leaves. I.B., immunoblotted with indicated antiserum. GPA1 or AGB1 were detected using anti-GPA1 (GPA1) or anti-AGB1 (AGB1) antibodies. Soluble and membrane-associated samples were run on the same gel and blot for direct comparison. The signal intensities are directly comparable (see Materials and Methods). Bands detected by the anti-GPA1 serum in the cytosol are non-specific as demonstrated by the observation that they are also present in the *gpa1*-4 null mutant sample. In the membrane fraction, the GPA1 protein runs as a split band under these conditions. Due to the low level of endogenous G protein subunits, sample overloading was necessary.

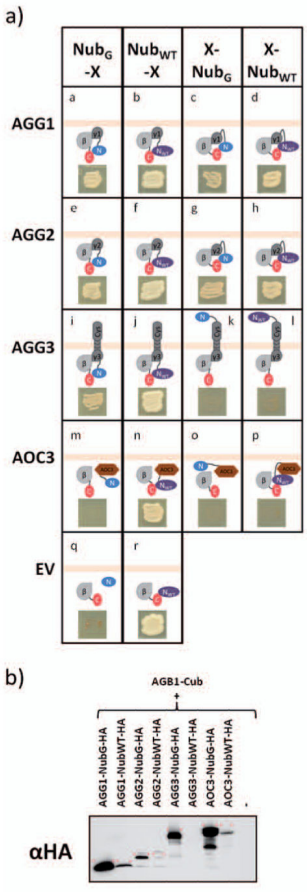

Figure 2. Membrane topology of AGG3

(a) Split ubiquitin assays between AGB1 and Gγ subunits. Yeast cells expressing AGB1 fused to the C-terminal half of ubiquitin (Cub) and AGG1, AGG2, AGG3, or AOC3 fused with the N-terminal half of the I13G mutant form of ubiquitin (Nub_G - weakened affinity to Cub) or fused with wild-type (WT) ubiquitin (Nub_{WT} - high affinity to Cub). An interaction was indicated by growth of diploid cells on interaction selective media containing 50 μM methionine. Orientations of the Nub fusions are indicated above each column, where X is AGG1, AGG2, AGG3, or the negative control AOC3 (as indicated to the left of each row). Schematic overviews of the different Gγ and AGB1 fusions are indicated above the yeast growth results. **(b)** Immunoblot analysis of $XNub_G$ and $X\text{-}Nub_{WT}$ fusions in panel **(a)**. Diploid cells were grown in SC –Trp –Leu –Met liquid media, and gel loading was normalized by cell density. Nub fusion proteins were detected with an anti-HA (αHA) antibody that binds the HA epitope on the C-terminus of the fusion protein. All Nub fusion proteins (marked with red *) were detected except AGG3-Nub_{WT}, which is consistent with the lack of interaction of that construct with AGB1-Cub, seen in **(a)** panel l. EV = empty vector.

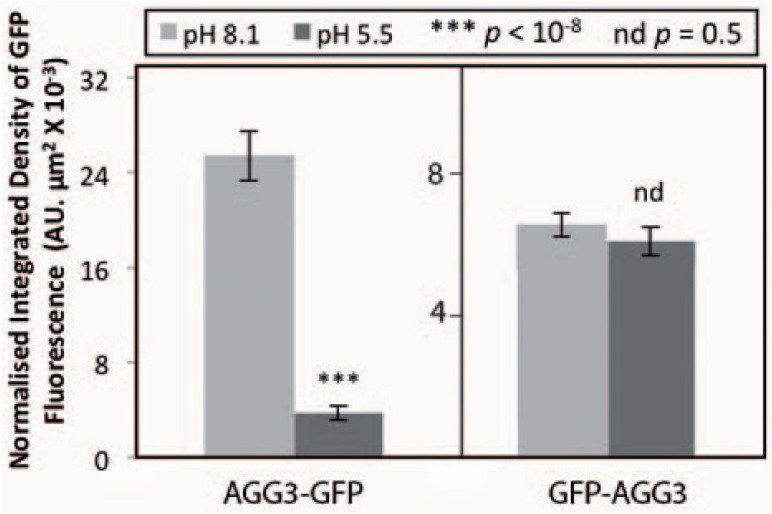

Figure 3. pH-dependent quenching of apoplastic GFP-fluorescence

Integrated density of fluorescence of GFP-AGG3 and AGG3-GFP stably expressed in Arabidopsis root cells normalized vs. autofluorescence observed in WT plants as discussed in the Experimental Procedures. Values represent the mean of over 200 individual cell measurements ±SEM.

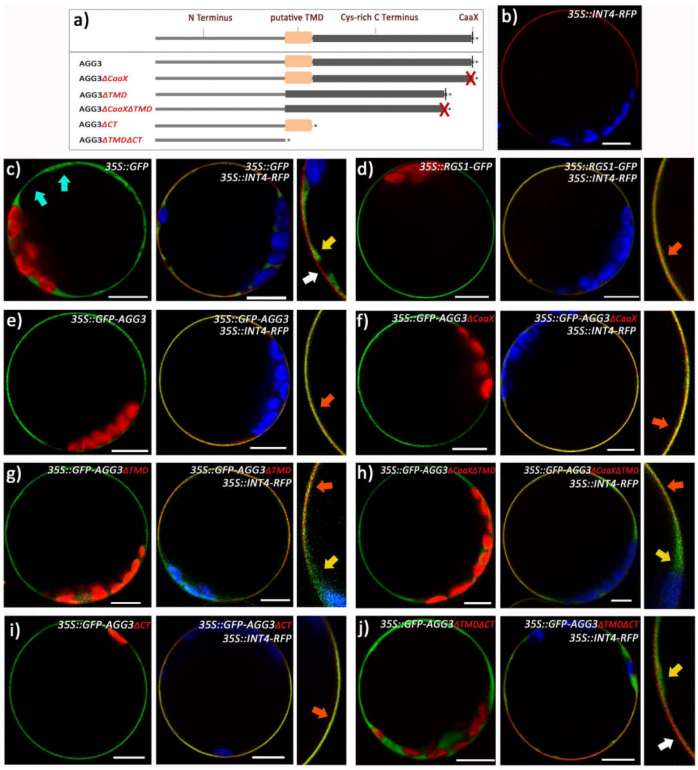

Figure 4. Subcellular localization of different AGG3 mutants in Arabidopsis mesophyll protoplasts

(a) Schematic overview of the various AGG3 mutants used for the localization studies. **(b)** Subcellular localization of the PM marker INT4-RFP in mesophyll protoplasts. **(c)** Protoplasts transiently over-expressing *GFP* alone (left) or in combination with *35S::INT4-RFP* (middle); a magnified section is depicted (right). **(d)** Protoplasts transiently over-expressing *RGS1-GFP* alone (left) or in combination with *35S::INT4-RFP* (middle); a magnified section is depicted (right). **(e)** to **(j)** Subcellular localization of AGG3 WT protein and different AGG3 mutants in protoplasts transiently over-expressing the constructs indicated on the top. Left: GFP signal of the different AGG3 GFP fusions; middle: co-localization studies with INT4-RFP; a magnified section of each picture in the middle is depicted on the right. All pictures show optical sections. GFP fluorescence is shown in green, RFP fluorescence in red; yellow signals indicate the complete merge of green and red fluorescence. Depending on the experiment, the autofluorescence of the chloroplasts is either depicted in red (pure localization of GFP fusions) or blue (co-localization studies with INT4-RFP). Blue arrows in **(c)** point to the typical fluorescence pattern observed in protoplasts caused by soluble fusion proteins. Yellow arrows in **(c)** and **(g)** to **(j)** highlight soluble fractions of respective GFP-fusions. White arrows in **(c)** and **(j)** indicate the lack of co-localization between the GFP fusions tested and INT4-RFP. Orange arrows in **(d)** to **(i)** indicate co-localization between the GFP fusions tested and INT4-RFP. Scale bar = 10 μm.

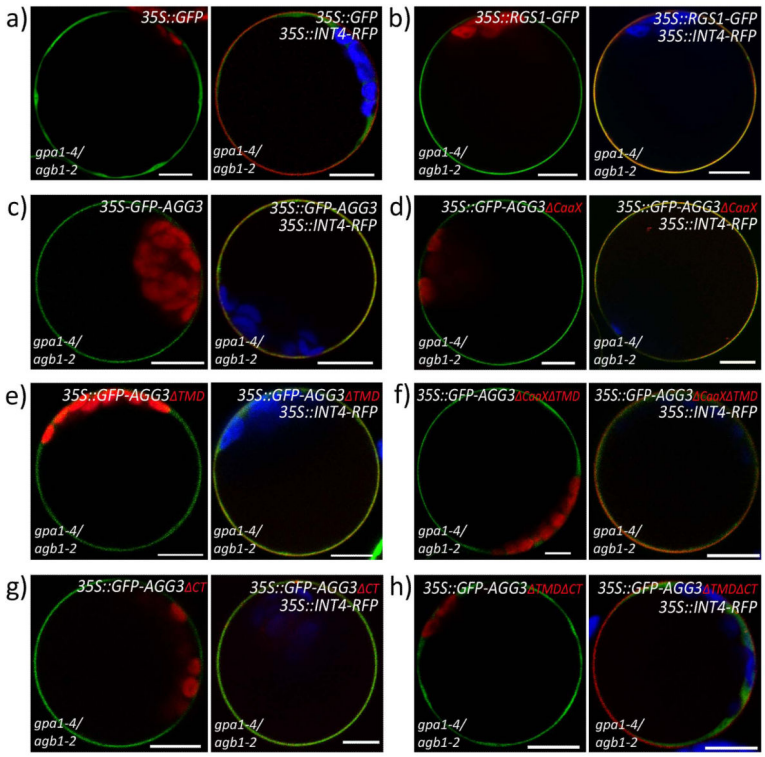

Figure 5. Subcellular localization of different AGG3 mutants in *gpa1-4/agb1-2* mesophyll protoplasts

(a) Protoplasts transiently over-expressing *GFP* alone (left) or in combination with *35S::INT4-RFP* (right). **(b)** Protoplasts transiently over-expressing *RGS1-GFP* alone (left) or in combination with *35S::INT4-RFP* (right). **(c)** to **(h)** Subcellular localization of AGG3 WT protein and different AGG3 mutants in protoplasts transiently over-expressing the constructs indicated on the top. Left: GFP signal of the different AGG3 GFP-fusions; right: co-localization studies with INT4-RFP. All images represent optical sections at the center of mesophyll protoplasts. GFP fluorescence is depicted in green, RFP fluorescence in red; yellow signals indicate the complete merge of green and red fluorescence. Depending on the experiment, the autofluorescence of the chloroplasts is either depicted in red (pure localization of GFP fusions) or blue (co-localization studies with INT4-RFP). Scale bar = 10 μm.